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SMUFD, D/A, 17 Feb 1972

TRANSLATION NO. 2370

DATE: Jan 6, 1969

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

URINARY HYDROXYPROLINE AND CIRCULATING HGH PRELIMINARY FINDINGS IN SOME ENDOCRINE DISEASES

Folia Endocrinologica (Endocrinological Folios), Vol 20, No 4, July-August 1967, pp 420-441

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During the approximately 50 years since the first research on urinary polypeptides (77), at least 47 different peptidic bodies have been identified in a rather voluminous and recent series of studies (8, 12, 16, 17, 73, 152, 178).

The volume of the excretion and the qualitative differences recorded under various physical and pathological conditions today seem to confer upon these nitrogenous products a rather significant value as an expression of the protein metabolism. During these past years, special interest was devoted to the study of the urinary excretion of hydroxyproline (15, 73, 93, 102, 116, 118, 131, 172).

Hydroxyproline is an iminoacid with a molar weight of 131.4; its rough formula is $C_5H_9O_3N$, whose structural configuration represents a 4-hydroxy-pyrrolidine-2-carboxylic acid. Discovered by Fischer in 1902 (42, 43), in the hydrolysis products of gelatin, it was isolated in the peptidic form in urine by Westall (172).

Hydroxyproline is present in the organism to a significant extent (13%) in the collagenous stratus, which as we know, constitutes about 1/3 of the body weight (125).

The iminoacid present in the collagen is not of direct exogenous, alimentary derivation; instead it comes from the hydroxylation of the proline such as it occurs through the conversion of lysin into hydroxyline (47, 62, 90, 106, 130, 138, 158, 164).

Many interpretative hypotheses have been set up as to the time and the way in which this process of hydroxylation takes place (25, 56, 59, 61, 62, 75, 113, 115, 119, 146, 163). Regardless of the metabolic phases of this conversion, it is certain that the transition from proline into hydroxyproline is considered a direct expression of the "turnover" of the collagenous tissue (48). The urinary peptides with hydroxyprolinic content as demonstrated by recent studies with proline C_{14}, therefore assume the role of indicators of this metabolic activity (56, 57, 134, 137, 176, 177, 186), while the rate of free iminoacid in the urine turns out to be rather small and insignificant (93, 118, 139, 86). Investigations conducted by various research groups in this connection revealed the relationships between hydroxyproline and the polypeptidic subunits which make up the collagen (89, 93, 114, 115, 118, 139, 186).

We know very well today that the first steps of collagen synthesis take place in the fibroblast (3, 44, 45, 51, 55, 58, 145): this is where the activation of the aminoacids takes place (113). The proline is hydroxylated into hydroxyproline and the lysin is converted into hydroxyline. Peptidic subunits up to 250 aminoacid, with a molar weight of about 30,000, accumulate in the ribosomes. A carbohydrate -- with an as yet unknown composition -- is added to the terminal residues of the aspartic acid, at the end of the peptide, and constitutes the link between the two peptidic chains (52). This is how the "alpha" chains are formed; each of them contains four of these peptides and has a molar weight of 120,000. Three "alpha" chains provided with weak bonds are then added to bring about the typical spiral structure of the tropocollagen with a molar weight of 360,000 (36, 37, 66, 68, 129, 153). The latter represents the fundamental complex of the collagen ("the basic building block")(147). Depending upon the progressive complexity of the intramolecular bond, three types of tropocollagen were identified: alpha, beta, and gamma (151, 154). In this phase, the tropocollagen is extracellular and it is soluble in neutral saline solution (154). By virtue of the strong bonds between contiguous chains, the tropocollagen successively loses its solubility characteristics and undergoes fibrillary metamorphosis. The massive stabilization of the fibrils and the constitution of the mature collagen would thus take place through covalent interfibrillary and intermolecular bonds between adjacent units of tropocollagen (52, 53, 54).

It has been suggested that a weak bond between the hydroxylic group of the hydroxyproline and the carboxylic group of a nearby chain could stabilize the collagenous fiber, in a manner similar to what occurs in the case of the disulfide bond of keratin (69, 70, 71). It is a fact that, as the molecular structure becomes increasingly complicated, the tropocollagen rates decrease, the solubility of the collagens is reduced, and the hydroxyprolinuria therefore shrinks (5, 14, 66, 89, 91, 154, 169, 186).

The presence of soluble collagen during the active growth phases has been traced to the more lively synthesis of the tropocollagen which would occur in such a quantity as to overcome the polymerization capacity of the

fibrils (66, 68). The metabolic inertia of the mass of insoluble collagen is well known indeed (167): the average life of the mature fiber is about 300 days (123), whereas that of "alpha" tropocollagen is about 5-24 hours (86, 89). In the normal adult, the collagen turnover would thus appear to be rather limited, which would indicate a "breakdown" under some particular conditions, that is, in the course of bone reformation [reshaping] and in the postpartum uterine involution (74, 122, 174). The catabolism of the insoluble collagen however would involve its conversion into tropocollagen and its successive hydrolysis into peptides and amino acids (84, 174).

On the basis of recent experimental investigations, it has been possible to ascertain that hydroxyprolinuria is sustained by the transition in the urine, of the imino acid into the state of metabolic precursor (active imino acid) or the state of catabolite of the collagen fiber or, likewise, of nonmetabolized alimentary hydroxyproline. The increase in hydroxyprolinuria would therefore be brought about by the increase in the anabolism of the collagen, by the stoppage of its polymerization (which occurs in osteolathyrism), or finally by increased tissue catabolism, as was also observed in extensive burns (48, 89, 98, 173). On the other hand, a reduction in hydroxyprolinuria would indicate a slowdown in the collagen metabolism due to a decrease in the processes of biosynthesis or perhaps due to an abnormal accumulation of insoluble collagens. Decreased excretion of immino acid would occur also because of a scurvy-producing diet (94, 114).

Many observations therefore seem to suggest that hydroxyprolinuria constitutes an indication proving the rate of metabolically-active collagen although in the current situation we cannot yet state specifically whether catabolic processes prevail or whether phenomena pertaining to the biosynthesis of the connective tissue predominate (48). A number of physiopathological conditions are very significant in this connection. In particular it has been observed that young animals, with a higher rate of soluble collagen and with an accelerated metabolic turnover, reveal increased hydroxyprolinuria (67, 114, 123, 124). It was also found that, in scurvy, where both the soluble collagen rate and the connective tissue synthesis decrease, a diminution of hydroxyprolinuria takes place (121). Finally it was observed that, in lathyrism, the dramatic increase in the soluble collagen is accompanied by a high urinary excretion of free and peptidic hydroxyproline. In this last-named condition, fibrillogenesis is in fact blocked in the tropocollagen stage (89, 105, 114, 160, 162).

The influence on hydroxyprolinuria, explained by hormones with osteometabolic action, such as the growth hormone and parathormone has been amply documented by various researchers (Bates, Dull, Keiser, Klein, Lee, Prockop, Smiley); it was thus possible to ascertain the existence of a significant relationship between urinary excretion of hydroxyproline and the turnover of tropocollagen (8, 32, 33, 92, 96, 97, 102, 144). Consequently, the study of the urinary excretion of hydroxyproline very recently turned out to be a new parameter in the evaluation of the turnover of the conjunctive tissues during growth and in some morbid conditions (32, 33, 35, 88,

89, 96, 98, 103, 107, 116, 117, 118, 143, 149, 150, 155, 159, 186).

In our research here, we have come to feel that an examination of the behavior of hydroxyprolinuria is worth-while; we conducted this study in a series of patients whose altered endocrinometabolic situation had produced some conditions capable of affecting hydroxyprolinuria to an appreciable extent. In particular, we considered the relationships between the urinary excretion of hydroxyproline and the concentration of circulating HGH in some patients with metabolic disorders of the diabetic type.

Material and Methods

The investigations were conducted on a total of 71 subjects. In particular, we examined 40 patients with diabetes mellitus, 11 patients with acromegaly, and 20 subjects who were clinically healthy.

The age of the diabetics, who were members of both sexes, was between 9 and 78 years; in 11 cases, the metabolic syndrome developed before the age of 20; in 4 patients, it assumed the clinical and endocrinometabolic characteristics of diabetes with hypophysary overtones.

All subjects examined were kept on a diet devoid of gelatin, fish or meat during the 24 hours prior to the time the urine samples were taken and throughout the entire time of the experiment. The urine samples were kept at a temperature of + 4° C under a layer of toluene.

The blood sample for the determination of the HGH was taken at base conditions on the second day of the experiment, about 8-10 hours after the last food intake.

In all patients subjected to insulin treatment, throughout the entire period of the experiment, administration of insular hormone was suspended.

To determine the plasmatic HGH rate, we used the radioimmunology method proposed by Hunter and Greenwood (80) and modified by Berson and associates (11), with some additional features suggested by us (156).

The determination of the total hydroxyproline rate in the urine was made according to the technique proposed by Prockop and Udenfriend (140) which employs the evaluation of a chromophore with an absorption spectrum on the wavelength of 560 mmu.

Results

The results of the determinations of hydroxyprolinuria and of the circulating HGH rate are given in Tables 1, 2, and 3.

Table 1. Normal Subjects

a N.	b Name	c Età a.	HCH mg%/ml	d Idrossiprolina urinaria mg/24h
1	S.N.	10	4,5	46
2	D..Gl.	9	7,8	52
3	V.R.	13	6,0	50
4	S.C.	11	5,8	43
5	C.M.	15	4,6	32
6	L.P.	12	6,3	30
7	S.A.	15	3,8	44
8	T.P.	15	5,6	38
9	M.G.	21	3,8	22
10	T.R.	28	4,1	26
11	D.M.	27	2,6	21
12	D.P.A.	40	2,0	16
13	A.C.	40	3,5	18
14	B.D.	56	2,5	15
15	C.N.	43	1,8	20
16	S.V.	19	3,9	28
17	P.F.	22	3,2	20
18	B.L.	21	4,0	14
19	G.T.	60	2,1	10
20	M.M.	20	3,0	16

Key: a. Number
b. Name
c. Age
d. Urinary hydroxyproline, mg/24 hr

The urinary excretion of hydroxyproline in normal subjects was between 10 and 52 mg/24 hr, with an average of 28.35 mg/24 hrs.

The hydroxyprolinuria registered in young subjects appeared greater than that registered in adults. Indeed, the daily elimination of imino acid varied between 30 and 52 mg/24 hours. These data agree with the observations made earlier by other researchers.

Table II. Diabetes Mellitus

a N.	b Nome	c Età	d Inizio: e Prima 22 a.	d Inizio: f Dopo 22 a.	g Intensità del diabete	h Acidosi	i Complicazioni	HGH mμg/ml	j Idrossiproline urinaria mg/24h
1	N.S.	37	+		+++	+	+	7.0	60
2	S.L.	13	+		+++	+		10.3	125
3	M.G.	9	+		+++	+	+	7.0	45
4	F.A.	13	+		+++	+		12.6	96
5	M.P.	17	+		+++	+		9.7	105
6	T.P.	13	+		+++	+		8.9	85
7	M.F.	26	+		+++	+	+	11.5	65
8	P.S.	24	+		+++			3.4	26
9	T.G.	41	+		+++		+	7.1	95
10	P.L.	28	+		++	+	+	6.8	120
11	S.I.	33	+		+			1.0	52
12	A.A.	43		+	+++	+	+	8.3	48
13	L.M.	55		+	+++	+	+	10.0	47
14	B.Z.	53		+	+++		+	6.0	52
15	S.A.	53		+	+++	+		12.0	36
16	T.D.	51		+	+++	+		8.3	28
17	C.M.	51		+	+++	+	+	5.0	80
18	E.A.	44		+	+++			1.2	33
19	M.P.	40		+	+++	+		2.8	11
20	C.A.	39		+	+++			3.3	40
21	S.A.	66		+	++		+	4.3	40
22	T.O.	53		+	++			4.0	70
23	F.P.	47		+	++		+	4.1	70
24	C.C.	50		+	++			4.3	13
25	P.A.	44		+	++			3.5	19
26	B.R.	78		+	++		+	4.8	38
27	P.E.	63		+	++		+	2.9	15
28	C.M.T.	45		+	++		+	3.4	16
29	P.A.	70		+	+			4.0	25
30	V.A.	26		+	+			5.0	20
31	D.F.	56		+	+		+	1.3	14
32	P.R.	50		+	+			3.8	18
33	S.A.	47		+	+			3.5	10
34	B.R.	35		+	+			9.6	62
35	M.O.	41		+	+			8.3	24
36	B.C.	51		+	+			5.3	50
37	C.A.	29		+	+		[illegible]	10.0	210
38	N.T.	57		+	+		[illegible]	6.8	240
39	F.E.	66		+	+		[illegible]	13.5	235
40	G.V.	30		+	++		[illegible]	13.3	225

Key: a. Number
b. Name
c. Age
d. Start
e. Before age 22
f. After age 22
g. Intensity of diabetes
h. Acidosis
i. Complications
j. Urinary hydroxyproline, mg/24 hrs

Table III. Acromegaly

Key: a. Number
b. Name
c. Age
d. Diabetes
e. Urinary hydroxyproline, mg/24 hrs

As regards the circulating HGH rate in the controls, this came out in the range of the norm and produced the highest values in growing youngsters (3.8-7.8 mmug/ml).

In diabetic subjects, the excretion of imino acid appeared greater than that registered in the controls, with an average value of 66.07 mg/24 hrs and a percentage increase of 133.15 percent (Figures 1 and 2). But if we consider the two groups of patients examined separately, then we can make some observations that are rather interesting. As a matter of fact, average hydroxyprolinuria turned out to be 79.45 mg/24 hrs in young diabetics and 61.0 mg in old diabetics: compared to the controls, this gave us a respective increase of 180.21 percent and 115.16 percent (Figures 2, 3, and 4). Hydroxyprolinuria thus turned out to be noticeably higher in young diabetics. On the other hand, the urinary excretion of hydroxyproline appeared particularly distinct in subjects where the diabetic syndrome was combined with clinical and humoral signs of increased somatotropinic incretion and in some acromegaly patients where metabolic disorders of the diabetic type could not be documented.

It thus appears evident that hydroxyprolinuria reveals the highest values in the hypophysary diabetic and in the young diabetic, that is, in cases where we often have a clinical picture of most severe diabetes, a picture that is sometimes unstable and that easily involves ketoacidosis.

The fact is that the average values recorded in these two groups of patients were 227.5 mg/24 hrs in hypophysary diabetes and 79.45 mg/24 hrs in youthful diabetes.

The concentration of HGH explored in a fasting state, under basic conditions, produced the highest values in the acromegaly patients and in some young diabetics. This last observation, reported earlier by Conti, and associates, has been confirmed once again in this series of investigations so that we have good reason to state that youthful diabetes--particularly if it is very severe--is associated quite frequently with HGH concentrations above normal.

As regards the relations between the two endocrinometabolic parameters studied in this investigation (Tables 2 and 3; Figures 2, 3, 4, and 5), we must emphasize that, in subjects with more pronounced hydroxyprolinuria, the circulating HGH concentration is often higher than the norm. This phenomenon is most evident in young diabetics (Figure 4) and in acromegaly patients with apparent diabetes: in this last group of patients, the daily excretion of hydroxyproline reaches the highest values and even the circulating HGH concentration appears exceedingly pronounced (Figure 5). However, while there is no doubt that correlations do exist between hydroxyprolinuria and the HGH rate, it must be pointed out very specifically that a strict and direct proportionality is not always recognizable between these two parameters. With equal circulating HGH concentration, we in fact observe that the hydroxyprolinuria is relatively higher in acromegaly subjects with diabetes than in young diabetics. This is obviously due to the intervention of other, not well known factors which further aggravate the protein exchange disorders in the disease of acromegaly.

Conclusions

The documented influence of hormones with osteometabolic action, such as HGH and parathormone, on hydroxyprolinuria caused us to consider bone collagen as the major source of hydroxyproline eliminated through the urine (8, 32, 33, 92, 96, 97, 102, 144). The relationships recorded between the soluble collagen rate and the urinary hydroxyproline seem to support the existence of a direct correlation between growth and hydroxyprolinuria. Cross, as a matter of fact, was able to demonstrate the existence of a precise relationship between the growth rate and the dimension of the soluble pool of collagen (66). Systematic research conducted by Jones and associates on subjects between the ages of 5 and 49 years demonstrated that hydroxyprolinuria increased from infancy to puberty; it reaches maximum values at the age of 14-15, with the excretion of 350-390 mg/24 hrs. After the age of 23, the rate of urinary excretion of hydroxyproline becomes stabilized around 30 mg/24 hrs, with daily variations of less than 10 percent. The research group of Ziff and associates was likewise able to determine an increased elimination of hydroxyproline in children in the growth phase and furthermore noted that the supply of HGH is accompanied by a definite increase in hydroxyprolinuria (72, 88, 186).

The relationships between the growth hormone and the deposit of collagen have been investigated for quite some time and the research conducted with Proline-C_{14} documented the way in which the capture of this immino acid, by the collagen, increases as a result of the supply of somatotropic hormone (4, 7, 26, 99, 142). As for the rest, it is well known that the important task of the growth hormone in the metabolic dynamics of the mucopolysaccharides suggested the perfection of a biological procedure for the determination of the GH (19, 20, 28, 50, 120).

In patients with acromegaly in the active phase, increased hydroxyprolinuria was found (9, 10, 31, 33, 80, 88, 168), whereas in nanosomia cases, the urinary elimination of imino acid turned out to be particularly small (33, 72, 81, 108, 132, 170). In a series of studies of various endocrine diseases, Benoit and associates, as well as Bonadonna and associates, were able to demonstrate that the excretion of hydroxyproline increases after stimulation with somatotropic hormone, gonadotropin and thyroid hormone; the effect resulting from parathormone turned out to be inconstant (9, 10, 13). These same authors investigated the urinary excretion of hydroxyproline in 12 diabetic patients between the ages of 18 and 62. Only in one case was hydroxyprolinuria considerably increased whereas in another observation it differed little from the high limits of the norm. It must be pointed out that, in both cases, diabetes had developed at an early age and that the patients examined were subjected to insulin treatment. This last disclosure is particularly important if we consider the well-known action of insulin upon the regulation of protein exchange in general and upon the utilization of the aminic acids (100,109, 111, 112, 157, 158, 179).

On the other hand, the pathogenic importance of GH in diabetes mellitus has for a long time been considered quite seriously and has been quite validly confirmed in experimental studies and clinical observations (1, 18, 24, 23, 29, 30, 40, 41, 76, 78, 81, 82, 110, 127, 141, 171, 180, 185).

In this connection, the dosage of somatotropic hormone in the plasma has led to interesting discoveries. Conti and associates (21, 22, 23) were able to show that the disorders in somatotropinic incretion are not foreign to endocrine disorders produced by an absolute or relative insulin deficiency. In particular, the concentration of circulating GH appeared significantly increased in youthful diabetes, especially when signs of incipient disturbance of the acid-base equilibrium are manifest. These observations, in agreement with the first discoveries of Forsman and Gerzell (46), were successively confirmed by Erlich and Randle (38, 39), Pfeiffer (128), and Greenwood (63, 65).

As regards the results obtained in this, our first preliminary research, we found that in patients with diabetes mellitus the urinary excretion of hydroxyproline appeared to be greater than the average recorded among the controls; this increase is more marked in youthful diabetes, in other words, where the metabolic disorder was more severe and unstable. On the

other hand, in patients whose acromegaly syndrome was complicated by obvious disorders in glycidic exchange, the excretion of imino acid appeared particularly pronounced: it was much greater than the--albeit very high--values that are typical of acromegaly not connected with diabetes mellitus. These discoveries become even more significant when we consider that, in the adult diabetic, the rate of hydroxyprolinuria turned out to be moderately higher than the average levels.

It seems to us therefore that we are justified in agreeing that metabolic disorders, as diabetes, also involve the collagen and that they are increasingly manifest--particularly at an early age--when the turnover of the fundamental substance is already physiologically increased with respect to the active body growth.

Our discoveries thus indicate that there is a significant relationship between hydroxyprolinuria and body growth. The fact is that in the young control subjects, in the young diabetics, and in acromegaly patients, in whom the HGH levels appeared highest, we found a noticeable increase in the urinary excretion of hydroxyproline.

It was demonstrated quite some time ago that the plasmatic levels of HGH are usually high in the youthful diabetes patient and it was assumed that hypersomatotropinism, in this morbid condition, was the expression of an early functional impairment of the hypophysis or rather the result of an increatory reaction of the secondary hypophysis to metabolic disorders, particularly to the limited utilization of glucose produced on the cell level by the insulin shortage. But, regardless of the underlying physiopathogenic significance of hypersomatotropinism in youthful diabetes, there is no doubt that this is one of the factors most actively involved in the cause for the increase in hydroxyprolinuria precisely because of this morbid condition.

From a general viewpoint, therefore, we can conclude that both diabetes mellitus and acromegaly represent two morbid conditions which definitely have an effect on the metabolism of collagen, to the point where they often trigger a distinct increase in hydroxyprolinuria. The metabolic disorder furthermore seems to become more severe when the two sickness pictures are combined so as to give rise to acromegalic diabetes.

This conclusion, in our opinion, cannot be challenged by the observation that, in some cases of diabetes and acromegaly, there is normal hydroxyprolinuria; there are many factors which, though they are not a part of the disease of diabetes and acromegaly, do become involved in the intimate economy of collagen.

Some authors, particularly Benoit and associates, as well as Bonadonna and associates, deny that there are any noticeable variations in hydroxyprolinuria in diabetes mellitus: but in this connection we want to point out that both groups of researchers conducted their investigations

primarily on adult diabetics who were kept under control with insulin, in other words, in subjects who were in a situation definitely different from that of the patients whom we examined.

The relationships between circulating HGH and hydroxyprolinuria appear to be somewhat more complex when we compare the results obtained in some young diabetics and in acromegaly patients with diabetes. In these two groups of patients, as a matter of fact, we cannot always recognize a strict correlation between the level of somatotrope and the volume of hydroxyprolinuria because, with an equal concentration of circulating GH, the maximum excretion of hydroxyproline is characteristic of acromegaly with diabetes. This obviously documents the fact that insulin shortage aggravates the metabolic disorder of collagen to the point where it promotes a subsequent noticeable increase in the urinary excretion of imino acid. We must not be astonished that, in acromegaly without any connected apparent disorders in glycidic exchange, the behavior of hydroxyprolinuria is often changeable and not strictly correlated with the level of circulating HGH: this fact likewise figures in the easy variation of the processes of bone reshaping, through the intervention of those rather disparate factors which are present during the deposit and reabsorption of collagen.

Summary

The relationships between the concentration of circulating HGH and the urinary excretion of hydroxyproline were studied in 71 subjects, including 40 with diabetes mellitus, 11 with acromegaly, and 20 who were clinically healthy.

The plasmatic HGH was dosed with the radioimmunoelectrophoretic method of Hunger and Greenwood and the determination of total urinary hydroxyproline was made according to the technique of Prockop and Udenfriend.

It was demonstrated that in patients with diabetes mellitus the urinary excretion of hydroxyproline is often greater than the average figures recorded in the controls. This increase is more marked in youthful diabetes, in other words, where the metabolic disorder is more severe and unstable. On the other hand, in patients in whom the acromegaly syndrome was complicated by obvious disorders in glycidic exchange, the excretion of the imino acid appears particularly pronounced: it is very much greater than the--by the way in themselves rather high--values that are typical of acromegaly not connected with diabetes mellitus.

It was found that there is a significant relationship between hydroxyprolinuria and body growth. Indeed, in the controls, in young diabetics, and in acromegaly patients, in whom the HGH levels are highest, we regularly find a noticeable increase in the urinary excretion of hydroxyproline.

However we did not always find a strict correlation between the plasmatic level of the somatotropic hormone and the urinary excretion of imino

acid, particularly in young diabetics in acromegaly patients with diabetes because--with an equal concentration of somatotropic hormone--the higher values of hydroxyprolinuria appeared characteristic of acromegaly with diabetes.

This discovery--according to the authors--documents the fact that, in diabetes mellitus, insulin shortage helps aggravate the metabolic disorder of the collagen.

It is concluded that diabetes mellitus and acromegaly are two morbid conditions which have a profound effect on collagen metabolism and that the metabolic disorder is aggravated when the two disease pictures are combined into acromegalic diabetes.

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FIGURE APPENDIX

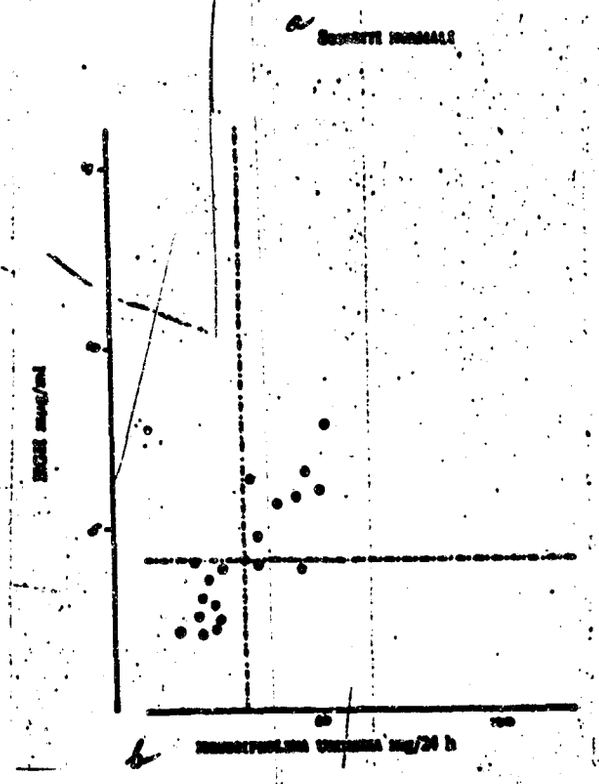

Figure 1.
Key: a. Normal subjects
b. Urinary hydroxyproline mg/24 hrs

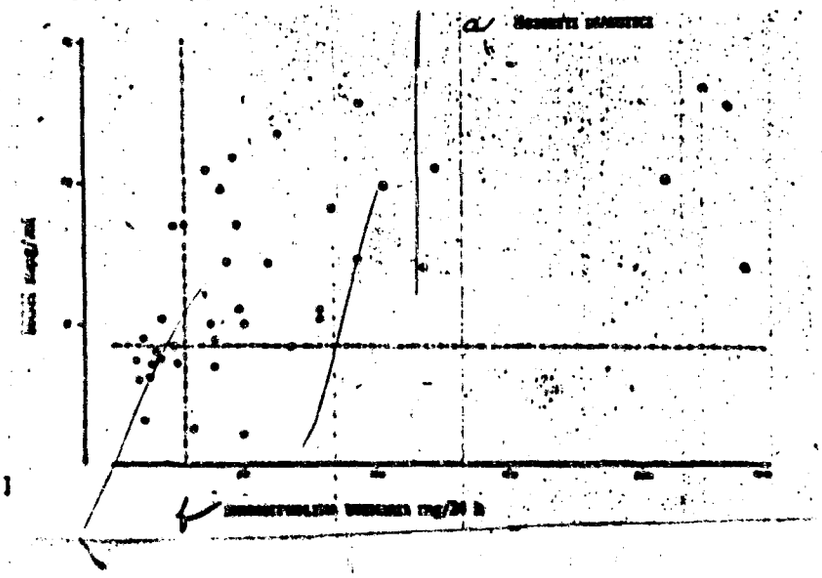

Figure 2. 1

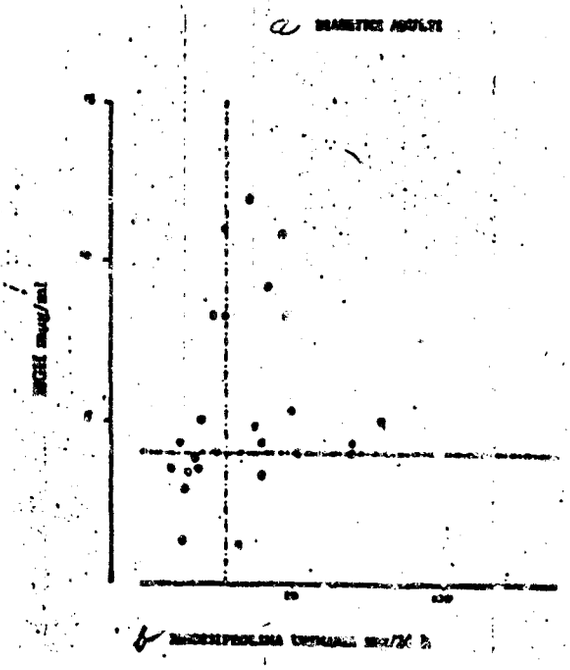

Figure 3
Key: a. Adult diabetics
b. Urinary hydroxyproline, mg/24 hrs

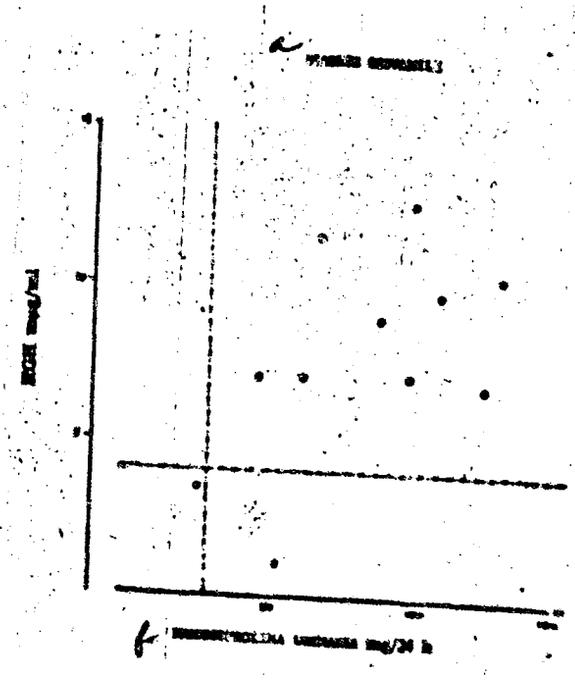

Figure 4. Key: a. Youthful diabetes
b. Urinary hydroxyproline, mg/24 hrs

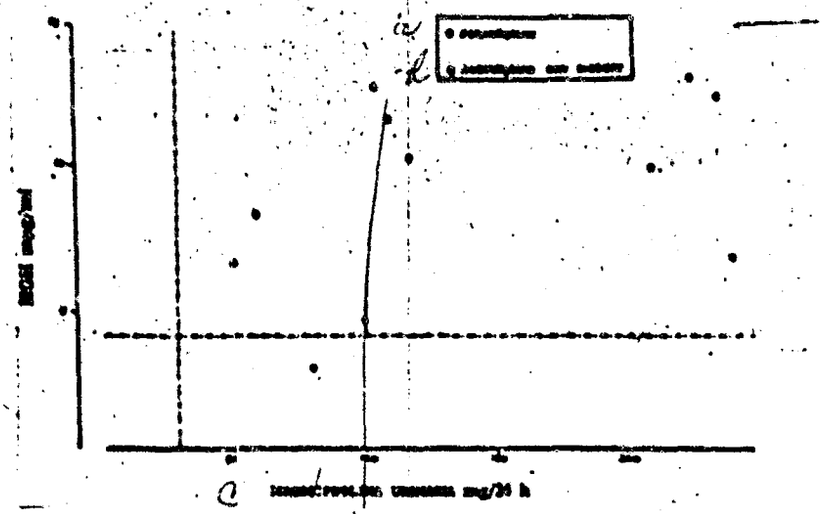

Figure 5.

Key: a. Acromegaly patients
b. Acromegaly patients with diabetes
c. Urinary hydroxyproline, mg/ 24 hrs.